EXHIBIT I

Reprinted from *J. Mol. Biol.* (1996) **262**, 21–30

JMB

Surface Display of Proteins on Bacteriophage λ Heads

Y. Gi Mikawa, Ichi N. Maruyama and Sydney Brenner

J. Mol. Biol. (1996) 262, 21–30

JMB

Surface Display of Proteins on Bacteriophage λ Heads

Y. Gi Mikawa[1], Ichi N. Maruyama[1]* and Sydney Brenner[2]

[1]*Department of Cell Biology The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla CA 92037, USA*

[2]*Department of Medicine University of Cambridge Hills Road, Cambridge CB2 2QQ, England*

**Corresponding author*

We have developed plasmid and phage vectors for the display of foreign proteins on the surface of bacteriophage λ capsid by modifying the *D* gene which encodes the major head protein gpD. The vectors have multiple cloning sites, and permit colour selection and conditional chain termination for recombinants. Displayed proteins can be fused to either the N or C terminus of gpD by a peptide linker. The conditional chain termination scheme, *via* a host *Escherichia coli* suppressor activity, allows the fusion and assembly of homomultimeric proteins as well as control of the number of fusion proteins per phage particle. We have successfully displayed β-lactamase, IgG-binding domains of the *Staphylococcus aureus* protein A, and β-galactosidase by cloning the genes into the vector. The constructs express functionally active proteins fused to gpD that assemble into phage particles. These results suggest that gpD may be fused to many other peptides and proteins at their N or C terminus and the fusion products may be accessible on the surface of bacteriophage λ particles.

Keywords: *D* gene; conditional fusion; affinity binding; phage display

Introduction

The fusion phage system introduced by Smith (1985) displays foreign peptides or proteins on the surface of filamentous bacteriophage particles *via* fusion to the phage coat proteins. This has been widely used to select particular clones from a large ensemble by construction of libraries of variant peptides, which can be searched for *in vitro* by affinity selection (Wells & Lowman, 1992). Other surface expression schemes have been devised for bacterial cells (Lu *et al.*, 1995) and for animal viruses (Kasahara *et al.*, 1994). However, all of these, including filamentous phage, require the fusion to be translocated across the plasma membrane, and there are many cytoplasmic proteins that cannot be extruded as fusion proteins. We previously reported a bacteriophage λ vector, λfoo, for the display of foreign proteins fused to the V protein, a major protein of the phage tail, which is more appropriate for the display of proteins that undergo folding in the cytoplasm (Maruyama *et al.*, 1994). The vector has been successfully used for the display of homo-multimeric proteins such as β-galactosidase and a plant lectin, BPA. Although these fusion constructs have been purified efficiently by panning on a microtiter plate coated with specific antibodies, the limitations of this system include the low expression level of fusion proteins, a few molecules per phage particle, and the low yields of phage after affinity purification, approximately 1%. In order to increase both the incorporation levels of fusion proteins and, hence, the recovery of fusion phage, we have further explored another λ phage coat protein, D protein, as the fusion partner of foreign proteins.

The D protein (gpD) has a molecular mass of 11 kDa and is essential for the stable phage head morphogenesis with wild-type genomes, although phages with less than 0.82 of the wild-type length can still be assembled in the absence of gpD. Structural analysis using cryo-electron microscopy has shown that gpD is assembled as trimers and appears as prominent protrusions on the capsid surface (Dokland & Murialdo, 1993). The conditional requirement for gpD in the phage capsid assembly and its location on the head surface have suggested that foreign proteins may be fused to gpD without interfering with phage assembly and the displayed proteins may be accessible for binding. During the course of this work, Sternberg and Hoess (1995) have reported a similar fusion system to the N terminus of gpD. Our system,

Abbreviations used: BSA, bovine serum albumin; gpD and gpV, λ phage proteins encoded by gene *D* and *V*, respectively; PCR, polymerase chain reaction; pfu, plaque-forming units.

described here, uses a conventional fusion scheme to either the N or C terminus of gpD and allows the assembly of multimer proteins into phage heads. The vectors have been designed to encode a long linker sequence consisting of proline and threonine repeats, an endopeptidase recognition sequence between gpD and foreign fusion proteins, and the *lacZ'* gene. The linker is to promote independent folding of the two domains, gpD and foreign proteins. The endopeptidase cleavage site allows infectious phage particles to be released from solid matrices under mild conditions. The *lacZ'* gene permits colour selection for recombinants in library construction.

Results

Modification of the *D* gene

In order to introduce a restriction site at the 5' end of the *D* gene, site-directed mutagenesis was carried out by the polymerase chain reaction (PCR) using λ1685 DNA as a template, an oligonucleotide primer encoding a *Kpn*I site between the second and third codons, and a reverse primer encoding an *Eag*I site after the termination codon of the gene. The PCR product was cloned into pACYC184, resulting in pλD1 (for details, refer to Materials and Methods and Figure 1), in which glycine and threonine were inserted between the second and third amino acid residues of gpD. To test the activity of the modified gpD, an amber mutant of the *D* gene, λF7 phage, was grown on an *Escherichia coli* host harbouring pλD1 and examined for EDTA sensitivity (for plasmid, phage and bacterial strains, see Table 1). In the absence of an *E. coli* amber suppressor, λF7 with an immature head lacking gpD is produced and is extremely sensitive to EDTA. In contrast, λF7 with gpD is resistant to EDTA treatment (Sternberg & Weisberg, 1977; Imber *et al.*, 1980). λF7 grown on EQ166, a suppressor-negative strain, was sensitive to EDTA treatment, while λF7 grown on EQ166 harbouring pλD1 showed weak EDTA resistance (Table 2). Although the activity of the modified gpD was very weak, this result indicated that gpD encoded by pλD1 could be supplied *in trans* and incorporated into phage particles. In order to raise the incorporation level of the modified gpD, we constructed a plasmid, pλD, using a high copy number plasmid vector, pBluescript II KS+ (pBSC). As shown in Table 2, λF7 grown on EQ166 harbouring pλD showed almost complete resistance to EDTA, which is equivalent to that of phage grown on Q358, a suppressor-positive strain.

Next, we examined the incorporation of gpD fusion products into phage particles by constructing plasmids, pλAD and pλLD, which encode chimaeric proteins, *Staphylococcus aureus* protein A and β-lactamase fused to the N terminus of gpD, respectively (Figure 1). These fusion proteins also made λF7 resistant to EDTA (Table 2), indicating that the fusion proteins could assemble onto λF7 heads. The fusion proteins expressed from a high copy number plasmid, pλAD, provided the phage with greater resistance than those from a low copy number plasmid, pλLD. This result indicated again that the expression levels were critical for the efficient incorporation of the fusion gpD into phage heads. Phage containing the fusion protein produced by pλAD was more sensitive to EDTA than that containing unfused gpD produced by pλD, suggesting either that the heads with the fusion proteins were not completely stable against EDTA or that the incorporation of the fusions was not as efficient as the unfused gpD.

Activity of proteins displayed on phage particles

To verify β-lactamase activity on phage particles, λF7 grown on bacteria containing pλLD were fractionated through a CsCl density-gradient and β-lactamase activity was assayed with Nitrocefin (O'Callaghan *et al.*, 1972) as a substrate (Figure 2). β-Lactamase activity co-migrated with phage particles through the gradient, indicating that active β-lactamase was successfully incorporated into phage particles. The activity of the IgG-binding domains of the protein A molecule fused to gpD was verified by adsorbing λF7 prepared from EQ166 harbouring pλAD to a microtiter plate coated with bovine serum albumin (BSA) after mixing the phage with rabbit anti-BSA antibody (Figure 3). The phage bound to the plate in an antibody concentration-dependent fashion but not to the plate without coated BSA. The results showed that the protein A domains on phage heads retained their IgG-binding activity. When grown on *E. coli* carrying pλAD, the λ2001 phage (Karn *et al.*, 1984) with the wild-type *D* gene also bound to the plate as efficiently as did λF7 (Figure 3), confirming that λ2001 captured the fusion proteins on its capsid even in the presence of the wild-type gpD.

Figure 1. Construction of vectors. (a) Flow-chart of vector construction. A map of the left arm of λ phage is shown at the top. The following abbreviations are used; MCS, multiple cloning sites; pBSC, pBluescript II KS + ; Δ, deletion; c, collagenase recognition sequence; f, factor Xa recognition sequence; PA, protein A; AI, *Apa*I; ALI, *Apa*LI; Ba, *Bam*HI; EI, *Eag*I; RI, *Eco*RI; HIII, *Hin*dIII; KI, *Kpn*I; NI, *Nhe*I; PI, *Pst*I; PII, *Pvu*II; SI, *Sma*I; SfI, *Sfi*I. (b) The squence between the second and third codons of the *D* gene on pλD5 and λfooDn is shown. The collagenase and factor Xa recognition sequences, and the unique restriction sites are indicated. (c) The sequence of the fusion junction in pλD3 and λfooDc. The sequences from the last codon, encoding 110th valine, of the *D* gene to *Eco*RI site in MCS are shown. The collagenase recognition sequence and the unique restriction sites are underlined. The ribosome-binding site of the *lacZ'* gene and the initiation codon methionine are indicated.

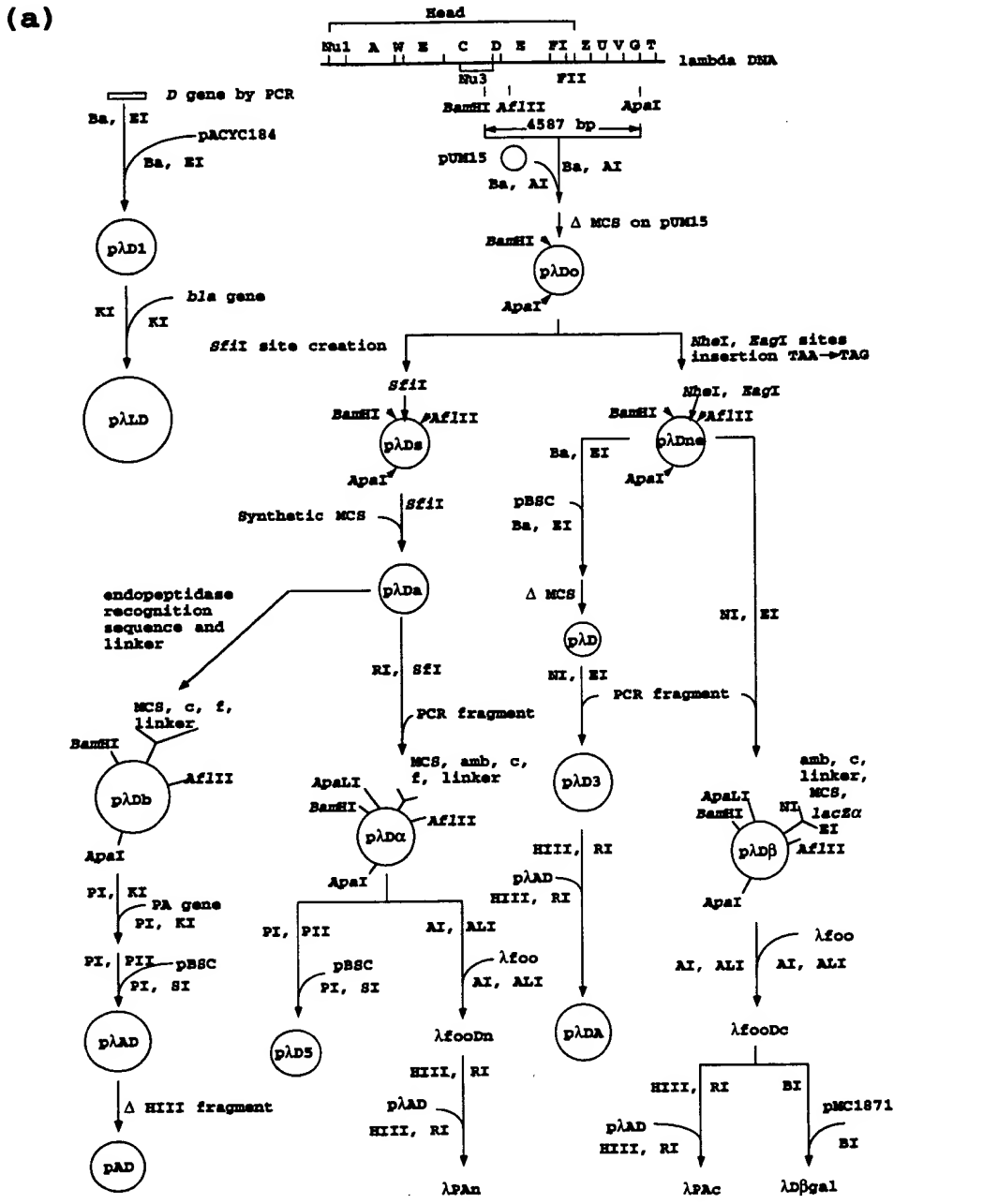
(a)
Head
Nu1 A W B C D E FI Z U V G T
lambda DNA
Nu3
FII
D gene by PCR
Ba, EI
pACYC184
BamHI AflII
ApaI
4587 bp
pUM15
Ba, AI
Δ MCS on pUM15
pλD1
pλDo
bla gene
KI
SfiI site creation
NheI, XagI sites insertion TAA→TAG
pλLD
pλDs
pλDne
Synthetic MCS
pBSC
Δ MCS
endopeptidase recognition sequence and linker
pλDa
pλD
RI, SfI
NI, EI
PCR fragment
MCS, c, f, linker
MCS, amb, c, f, linker
amb, c, linker, MCS, lacZα
pλDb
pλDα
pλDβ
pλD3
PI, KI
PA gene
PI, PII
pBSC
PI, SI
AI, ALI
λfoo
HIII, RI
pλAD
pλD5
λfooDn
λfooDc
Δ HIII fragment
pλDA
pMC1871
BI
pAD
λPAn
λPAc
λDβgal
(b)
HindIII
BamHI
SacI EcoRI
NotI
5'ATGACGGCCCCCGCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCAGCGGCCGCATAG
M T A P A S L H A C R S T L E D P R V P S S N S A A A amb
D1 D2
CCGACCGGGCCAAATTCTATCGAAGGTCGTGGGACTCCGACCCCGACCACTCCCACCCCGACTCCCACCCCGACCCCGACCCCG
P T G P N S I E G R G T P T P T T P T P T P T P T P T P T P
collagenase
factor Xa
ACTCCGACCGTTGGGCCAATTGTCACACAGGAAACAGCTATGACCATGATTACGGCCCCCGGGGCCAGCAAA
T P T V G P I V T Q E T A M T M I T A P G A S K
D3 D4
(c)
NheI
5'GTTTAGGCTAGCAGCTGGCCTGTTGGGCCCACTCCGACCCCGACCACTCCCACCCCGACTCCCACCCCGACCCCGACCCCGACT
V amb A S S W P V G P T P T P T P T T P T P T P T P T P T P T
D110
collagenase
HindIII
CCGACCGTTGGGCCAATTGTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAG
P T V G P I V T Q E T A M T M I T P S L H A C R S T L E
BamHI
SacI EcoRI
GATCCCCGGGTACCGAGCTCGAATTCGGCACTGGCC
D P R V P S S N S A L A

Figure 1.

Table 1. Plasmid, phage and bacterial strains

Strains	Relevant properties	Source/Reference
A. *Plasmid*		
pλD	Wild-type *D* gene in pBSC	This work
pλD1	Wild-type *D* gene in pACYC184	This work
pλDo	λ DNA in pUM15	This work
pλDs	*Sfi*I site in pλDo	This work
pλDne	*Nhe*I and *Eag*I sites in pλDo	This work
pλDa	MCS[a] in pλDs	This work
pλDb	Linker sequence in pλDa	This work
pλDα	Linker sequence in pλDa	This work
pλDβ	Linker and *lacZ'* in pλDne	This work
pλD5	Vector for fusion to N terminus of gpD	This work
pλD3	Vector for fusion to C terminus of gpD	This work
pλLD	φ(β-Lactamase-gpD)[b] in pACYC184	This work
pλAD	φ(*S. aureus* protein A-gpD) in pBSC	This work
pλDA	φ(gpD-protein A) in pBSC	Chang *et al.* (1978)
pACYC184	Low copy number plasmid	STRATAGENE
pBSC	pBluescript II KS+, high copy number plasmid	Casadaban *et al.* (1983)
pMC1871	Construct containing *E. coli lacZ*	Nilsson *et al.* (1985)
pRIT5	Construct containing *S. aureus* protein A gene	Yanisch-Peron *et al.* (1985)
pUC18	Construct encoding β-lactamase	Maruyama & Brenner (1992)
pUM13	Derivative of pBSC	I. Maruyama
pUM15	Derivative of pUM13	
B. *Phage*		
λ1685	Wild-type gpD	Maruyama *et al.* (1994)
λ2001	Wild-type gpD	Karn *et al.* (1984)
λF7	*imm21 cIts Dam15*	S. Brenner
λβgal	φ(gpV-β-galactosidase)	Maruyama *et al.* (1994)
λfoo	Vector for fusion to C terminus of gpV	Maruyama *et al.* (1994)
λfooDn	Vector for fusion to N terminus of gpD	This work
λfooDc	Vector for fusion to C terminus of gpD	This work
λPAn	φ(protein A-gpD)	This work
λPAc	φ(gpD-protein A)	This work
λDβgal	φ(gpD-β-galactosidase)	This work
C. *E. coli*		
EQ166	*sup*^{0}, a suppressor-negative strain	Maruyama *et al.* (1994)
MC8	*supG*	Maruyama *et al.* (1994)
Q358	*supE*	Maruyama *et al.* (1994)
TG1	*supE*	Maruyama *et al.* (1994)
WXO	*supF*	S. Brenner

[a] Multiple cloning sites.
[b] φ(A-B) indicates fusion of A to the N terminus of B.

We constructed also plasmid vectors for fusion to the C terminus of gpD. PCR reaction was designed to introduce *Nhe*I and *Eag*I restriction sites immediately after the termination codon of the *D* gene, resulting in pλDne (Figure 1). This PCR mutagenesis also replaced the original ochre stop codon with an amber codon for the conditional fusion through a host suppressor activity. From pλDne, pλD3 was created through pλD by inserting a PCR fragment encoding a collagenase cleavage site, proline and threonine repeats, multiple cloning sites, and *lacZ'*. In order to test the vector pλD3 for

Table 2. EDTA sensitivity of λF7 phage with wild-type or modified gpD

E. coli host	Plasmid[a]	Fusion product	Recovery (% of input phages[b])
EQ166 (*sup*^{0})	—	—	0.0
Q358 (*supE*)	—	—	116.7
EQ166 (*sup*^{0})	pλD	gpD	91.3
EQ166 (*sup*^{0})	pλD1	gpD	7.3
EQ166 (*sup*^{0})	pλAD	Protein A-gpD	27.3
EQ166 (*sup*^{0})	pλLD	β-Lactamase-gpD	0.6

[a] pλD and pλD1 encode unfused gpD; pλAD, fusion of IgG-binding domains to the N terminus of gpD; pλLD, fusion of β-lactamase to the N terminus of gpD. Plasmid pλD and pλAD were constructed from a high copy number vector, pBSC; pλD1 and pλLD were from a low copy number vector, pACYC184.
[b] Approximately 10^7 pfu (plaque-forming units) phages were treated with EDTA as described in Materials and Methods. Data are the mean of three independent measurements.

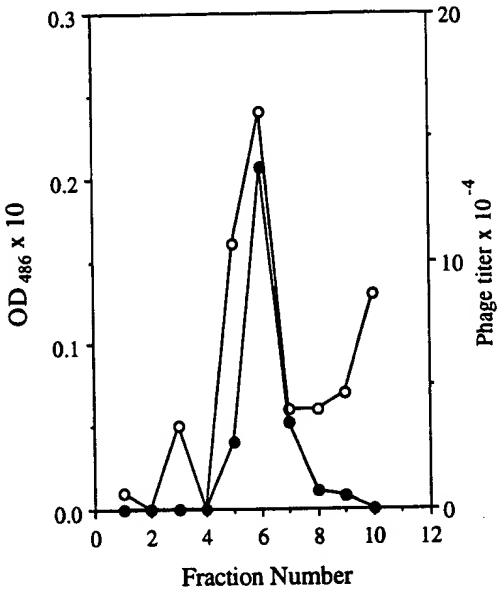

Figure 2. Co-migration of β-lactamase activity and phage particles. λF7 phage lysate from EQ166 harbouring pλLD was fractionated through a CsCl step-gradient and each fraction was titrated for phages (●) and β-lactamase activity (○). pλLD encodes the β-lactamase-gpD fusion product.

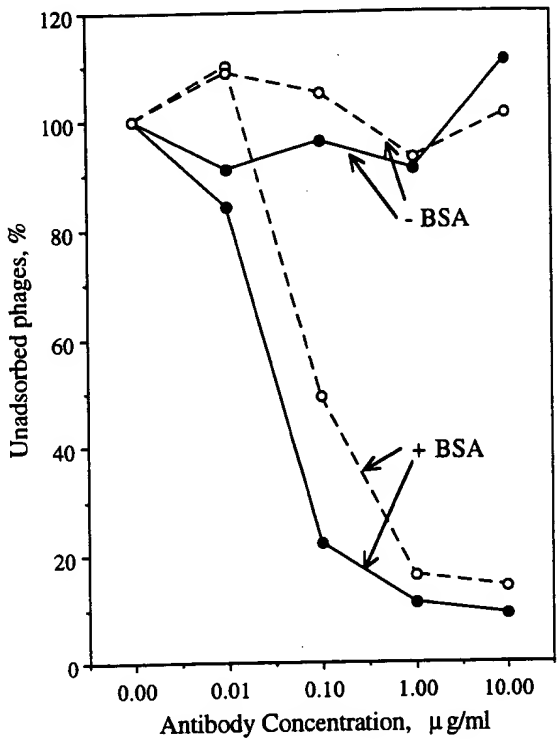

Figure 3. Adsorption of fusion phages to a microtiter plate. The IgG-binding activity of protein A domains fused to the phage particles was examined by its ability to bind to anti-BSA antibody. λF7 phage (○) and λ2001 phage (●) lysates prepared from EQ166 harbouring pλAD were incubated with anti-BSA antibody and were applied to a microtiter plate coated with BSA. After incubation, the supernatant was titrated for unadsorbed phages.

C-terminal fusion of gpD, a *Hin*dIII-*Eco*RI fragment, encoding the IgG-binding domain of *S. aureus* protein A, was excised from pλAD and cloned into pλD3 to create pλDA, which encodes protein A fused to the C terminus of gpD. The pλDA plasmid should produce both fused and unfused gpD in the presence of an amber suppressor of *E. coli* hosts such as Q358 and TG1. λF7 and λ2001 phages grown on TG1 containing pλDA bound to anti-BSA antibody, and were adsorbed to a microtiter plate coated with BSA (Table 3). This suggested that the fusion product was incorporated into λF7 and λ2001 phage heads together with unfused gpD.

Recovery of phage bound to solid matrices

The plasmid fusion vectors described above were designed to encode endopeptidase cleavage sites at their fusion junction (Figure 1(b) and (c)) for the efficient recovery of bound phages from solid matrices under mild conditions. λF7 or λ2001 grown on EQ166 harbouring pλAD or pλDA was adsorbed to a BSA-coated microtiter plate after mixing with anti-BSA antibody and bound phages were recovered as infectious phages from the plate by collagenase digestion (Table 3). Higher recovery of phages carrying both fused and unfused gpD (λ2001, and λF7 grown in TG1 harbouring pλDA) was observed than that of phage carrying only fusion gpD (λF7 grown on EQ166 harbouring pλAD). This is reasonable because phages with more fusion molecules may be released less efficiently by collagenase digestion from the plate. We also tried to recover bound phages by the addition of bacteria directly to the wells for infection and found that this method was constantly ten times less efficient than collagenase extraction (data not shown). The pλAD phage also contains the recognition sequence for the factor Xa endopeptidase (Figure 1(b)) but this enzyme failed to release phage from the plate. However, phages treated with factor Xa prior to incubation with

Table 3. Phage elution by collagenase

E. coli host	Plasmid[a]	Antibody	Recovery[b] (pfu) λF7	λ2001
EQ166 (*sup*^{0})	pλAD	+	2400	17,000
EQ166 (*sup*^{0})	pλAD	–	0	0
TG1 (*supE*)	pλDA	+	40,000	8000
TG1 (*supE*)	pλDA	–	0	0

The 2×10^7 pfu phages (100 μl) were mixed with or without 1.0 μl of anti-BSA antibody (0.22 μg/μl) and were bound to microtiter wells coated with BSA. Phage bound to the wells were eluted by collagenase treatment as described in Materials and Methods, and titrated on WXO.

[a] pλDA produces both unfused gpD and fused gpD to the N terminus of the IgG-binding domain of protein A, and pλAD produces only a fusion product of the domains fused to the N terminus of gpD since it has no amber stop codon at the fusion junction.

[b] Data are the mean of three independent measurements.

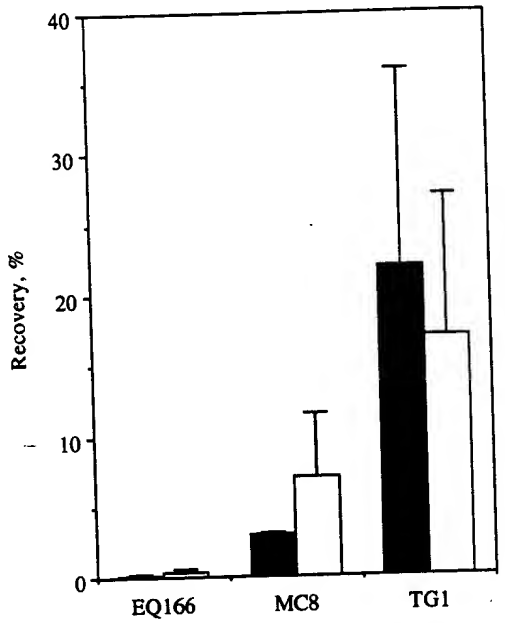

Figure 4. Binding of phages grown on bacteria with various suppressor activities. λPAn (filled bars) and λPAc (open bars) phages were grown on the bacteria indicated and partially purified by PEG precipitation. Phages, approximately 1.0×10^7 pfu, were applied to microtiter wells coated with rabbit anti-BSA antibody. Bound phages were eluted by collagenase treatment and titrated on WXO. The data are the mean ± SD of three separate measurements.

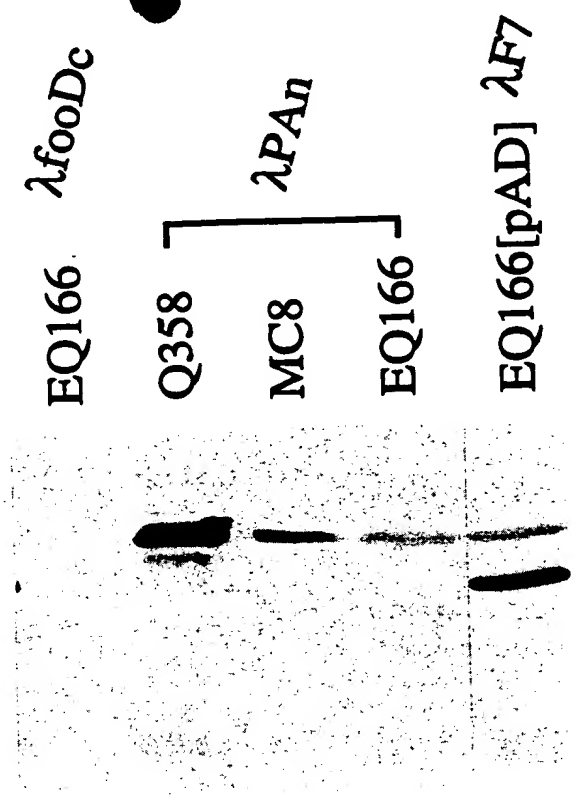

Figure 5. Western blot of the gpD product incorporated into phage particles. λPAn were grown on *E. coli* strains EQ166, MC8 or Q358, which have no, weak or strong suppressor activity, respectively, and partially purified by precipitation with PEG. Phage proteins were separated by gel electrophoresis, blotted onto a nitrocellulose filter, and stained with anti-BSA antibody as described in Materials and Methods. As references, λfooDc vector without an insert (left) and λF7 grown on EQ166 harbouring pAD (right) are shown. The plasmid pAD encodes the IgG-binding domain B fused to the N terminus of gpD. The bands on the lanes of λPAn grown on Q358 and MC8 correspond to a protein A IgG-binding domain B-gpD fusion product with a molecular mass of 33.0 kDa; the difference between the molecular masses of the fusion proteins encoded by λPAn and pAD is due to the linker sequences on λPAn. A lower band on the λF7 lane corresponds to protein A IgG-binding domain B fused to the N terminus of gpD, whose molecular mass is 30.6 kDa, close to the estimated value of 29.2 kDa. The faint band on the lanes of λF7 and λPAn grown on EQ166 is an unknown protein that is co-purified with protein A and cross-reacts with either primary or secondary antibody.

antibody were less efficiently adsorbed (2.7%) to the plate than those untreated (38%). This suggested that the factor Xa sequence could serve as an enzyme cleavage site between protein A and gpD but could not be accessed by the enzyme in the phage-protein A-antibody complex attached to the plate, probably due to steric hindrance.

Phage vectors

λ phage vectors should be more suitable than plasmid vectors for construction of complex libraries such as cDNA libraries. We constructed also two phage vectors, λfooDn and λfooDc, by replacing the *Apa*I-*Apa*LI fragment of λfoo with the modified *D* genes on pλDα and pλDβ, respectively (Figure 1). pλDα and pλDβ were derived from pλDa and pλDne, respectively, by inserting a PCR product encoding proline and threonine repeats of λfoo (for details, refer to Materials and Methods). To examine the incorporation of fusion proteins encoded by the phage vectors into their heads, the IgG-binding domain of *S. aureus* protein A was fused to the N terminus of gpD using λfooDn and to the C terminus of gpD using λfooDc, resulting in λPAn and λPAc, respectively. These phages were grown in bacteria having various suppressor activities and the IgG-binding activity of the phages was verified by adsorption of the phages pre-mixed with anti-BSA antibody to microtiter wells coated with BSA. As shown in Figure 4, the binding of the fusion phage was proportional to the suppressor activity of *E. coli* hosts used for the phage culture. When a strong suppressor strain, such as TG1, was used for phage culture, binding of the phage to the plate was more efficient than that of phages grown on a weaker strain, such as MC8. These results indicated that the IgG-binding domain of protein A was successfully displayed with IgG-binding activity on the surface of the phage particle. The fusion was further substantiated by western blots of purified phage particles, which were probed with anti-BSA antibody (Figure 5). Fusion proteins

Table 4. Affinity selection of λPAn and λPAc fusion phages

Input		Output		Yield (%)[a]	Enrichment factor[b]
λPAc	λβgal	λPAc	λβgal		
270	1.1×10^8	275	150	102	7.5×10^5
2700	9.3×10^7	1161	240	43	1.7×10^5
270	9.7×10^6	54	43	20	4.5×10^4
2700	8.7×10^6	864	63	32	4.4×10^4
λPAn	λβgal	λPAn	λβgal		
420	9.3×10^7	378	75	90	1.1×10^6
4200	9.3×10^7	4116	75	98	1.2×10^6
420	7.5×10^6	357	34	85	1.9×10^5
4200	8.2×10^6	3738	38	89	1.9×10^5

λPAn and λPAc phages grown on Q358 were respectively mixed with λβgal in various ratios indicated and were selected through a single round of affinity selection using a microtiter plate coated with rabbit anti-BSA antibody. Extracted phages with collagenase were titrated on EQ166 in the presence of X-gal. In this experiment, λβgal was used as an internal reference, since it formed blue plaques and could be distinguished from white λPAc or λPAn plaques. Data are the mean of three independent measurements.

[a] Yields of λPAc and λPAn are as the ratio of output to input.

[b] Enrichment factors are calculated by the ratio of output ratio, λPAc/λβgal, to input ratio, λPAc/λβgal.

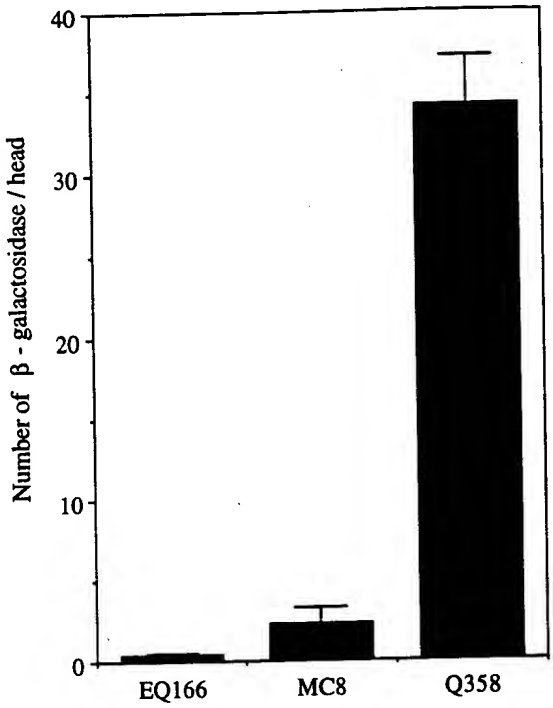

Figure 6. Estimated number of β-galactosidase molecules per phage particle. The numbers are estimated from relative enzyme activity of purified λDβ-gal phage particles, grown on the *E. coli* host indicated, to that of λβ-gal phage grown on MC8. Data are the mean ± SD of three separate preparations.

migrated as a band having molecular mass of 33.0 kDa, which was close to the calculated molecular mass of 32.0 kDa. The amount of fused protein A to phage capsids depended on the *E. coli* strains used for phage growth, consistent with the results that binding activity of fusion phages was highly dependent on suppressor activity of bacterial hosts.

Using the λPAn and λPAc phage constructs, an affinity selection experiment was carried out to examine how efficiently the phages were purified over a reference phage, λβ-gal (Table 4). Both the phages λPAn and λPAc were very efficiently purified with antibody immobilized on a microtiter plate by a single round of affinity selection. While the enrichment factor was equivalent to that obtained with λfoo fusion phages (Maruyama *et al.*, 1994), recovery yields of the phages were 10 to 100 times better than those obtained with λfoo fusion phages. These very high yields suggested that a small number, 100 or less pfu, of phage of interest could be affinity-purified from libraries containing a very large number of variants, such as a cDNA library or random peptide library.

In order to estimate the number of foreign molecules displayed on the phage surface, we fused β-galactosidase to the C terminus of gpD using λfooDc and termed the construct λDβ-gal. A similar construct using λfoo, λβ-gal, was analysed by electron microscopy to quantify the number of β-galactosidase molecules and the result indicated ~0.5 molecule of the enzyme, on average, per phage particle (Maruyama *et al.*, 1994). Therefore, by direct comparison of the β-galactosidase activity of purified λDβ-gal particles to that of λβ-gal, the mean number of the enzyme molecules incorporated into a phage capsid was estimated for λfooDc. As shown in Figure 6, the mean number of β-galactosidase molecules displayed on a phage capsid was 34.3 for λDβ-gal grown on Q358, which has a strong suppressor activity, while 2.3 for λDβ-gal grown on MC8, which has a weak suppressor activity. These results suggested that the incorporation of β-galactosidase molecules fused to gpD into phage heads was more efficient than that obtained with gpV fusion into phage tails.

Discussion

We have constructed vectors that allow fusion of proteins to either the N or C terminus of the D protein (gpD) of bacteriophage λ, permitting efficient display on the surface of the phage head. This shows that both ends of the gpD polypeptide chain are exposed on the external surface of the trimer. The ends are neither at the trimer interaction face nor at the surface that interacts with the E protein, the major head protein. This is an important result for which no guarantee existed at the start of this work.

Most phage display systems involve fusion at the N terminus of phage proteins although C-terminal fusions have been made for the *V* gene (Maruyama *et al.*, 1994; Dunn, 1995) and filamentous phage protein VI (Jespers *et al.*, 1995). For C-terminal fusions with gpD, we have employed the same linker as previously used with λfoo. This encodes

an endopeptidase cleavage site, long proline-threonine repeats, a ribosome-binding site, and a methionine initiator codon. Therefore, the vector expresses both fused and unfused gpD in various ratios depending on the suppressor tRNA activity of *E. coli* hosts to be used. The linker of proline-threonine repeats is unlikely to interfere with independent folding of the two distinct domains, gpD and foreign proteins. The endopeptidase recognition sequences between the two domains can be enzymatically cleaved, allowing infectious phage particles to be released from solid matrices under mild conditions.

Both of the plasmid vectors (pλD5, pλD3) and phage vectors (λfooDn, λfooDc) should be useful for the display of peptides or proteins. However, the C-terminal fusion vectors (pλD3 and λfooDc) may be more suitable for expression of cDNA because of the existence of many stop codons in the 3' untranslated region. For the expression of DNA-binding proteins, the N-terminal fusion vectors (pλD5 and λfooDn) may be more appropriate since many DNA-binding proteins such as Fos or lambda repressor have the binding sites at their N-terminal regions and the N-terminal fusion vector fuses the C terminus of the DNA-binding domain to the N terminus of gpD. Sternberg and Hoess's N-terminal D fusion display phage system requires the *loxP-cre* site-specific recombination-mediated incorporation from plasmid library and lambda transduction steps (Sternberg & Hoess, 1995). The phage vectors λfooDn and λfooDc described here can be used directly to construct large libraries of variant peptides and proteins. Using these vectors, the ratio of fused gpD molecules to unfused on the phage head can be controlled by the suppressor tRNA activity of *E. coli* hosts. This is an important feature for the expression of foreign proteins that may disturb the phage assembly. Without this feature, such clones might be under-represented in libraries.

EDTA-resistant phage could be recovered from λF7 phage grown on bacteria producing only completely fused gpD (Table 2), suggesting that almost the maximum number of molecules, ~400, of a fusion gpD can be incorporated into the phage particle. This high level of incorporation could be useful for affinity purification of particular clones from a library and for other experiments, such as raising antibodies against the displayed protein (Willis *et al.*, 1993) and gene delivery (Hart *et al.*, 1994).

Materials and Methods

Construction of D fusion vectors

In order to modify the 5' end of the *D* gene, a pair of oligonucleotide primers, 5'-GGATCCGGGGGTATTAA-TATGACGGGTACCAGCAAAGAAACCTTTACC and 5'-ATCGGCCGGTCGACTTAAACGATGCTGATTGC, were used for PCR amplification of the gene (Saiki *et al.*, 1988) using λ1685 DNA as a template. The former primer contains the ribosome-binding site of the *S. aureus* protein A gene (Uhlén *et al.*, 1984). The PCR fragment digested with *Bam*HI and *Eag*I was cloned between *Bam*HI and *Eag*I of pACYC184, resulting in pλD1.

Separately from the above construction, a 4587 bp *Bam*HI-*Apa*I fragment of λ1685 DNA encompassing the genes *'C, 'Nu3, D, E, FI, FII, Z, U, V* and *G'* was also cloned into pUM15 for the mutagenesis of the *D* gene, resulting in pλDo. The pUM15 plasmid is a derivative of pUM13 (Maruyama & Brenner, 1992) and has *Apa*I and *Bam*HI sites in the multiple cloning site (MCS). In order to create a unique *Sfi*I site between the second and third codons of the *D* gene, two pairs of oligonucleotide primers, 5'-CCGGGGATCCTCAACTGTGAGGA and 5'-ATGGCCCCGGGGGCCGTCATAAACATCCCTTACA-CTG, and 5'-TTGGCCCCCGGGGCCAGCAAAGAAAC-CTTTACCCATTA and 5'-TGCCCTTAAGCACGGCAG-AAACT, were used for PCR amplification of the gene. To create pλDs, the two PCR fragments digested with *Bam*HI and *Sfi*I, and *Sfi*I and *Afl*II, respectively, were cloned in tandem between the *Bam*HI and *Afl*II sites of pλDo. After eliminating all the remaining MCS of pUM15, a new MCS cassette encoding *Hin*dIII, *Sph*I, *Pst*I, *Acc*I, *Hin*cII, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sac*I, *Eco*RI and *Hae*III was introduced into the unique *Sfi*I site located at the 5' end of the *D* gene on pλDs, resulting in pλDa. A synthetic cassette encoding a collagenase recognition sequence (Germino & Bastia, 1984), factor Xa recognition sequence (Maina *et al.*, 1988) and linker sequence was cloned into the *Kpn*I and *Sfi*I sites in pλDa, creating the plasmid pλDb. The plasmids described above were used for the fusion of β-lactamase or the IgG-binding domain of protein A to the N terminus of gpD. After confirming that the fusion proteins were functionally active and were incorporated into the phage particle, the pλD5 vector was constructed by the following procedures. The linker sequence of λfoo (Maruyama *et al.*, 1994) was amplified by PCR with primers, 5'-GAATTCAGCGGCCGCATAG-CCGACCGGGCCAAATTCTATCGAAGGTCGTGGGA-CTCCGACCCCGACCACTCCC and 5'-AATGGCCCC-GGGGGCCGTAATCATGGTCATAGC. The former primer contains a *Not*I site, amber stop codon and endopeptidase recognition sequence. After digesting with *Eco*RI and *Sfi*I, the PCR fragment was cloned into pλDa to produce pλDα. A fragment containing the modified *D* gene was cut out with *Pst*I and *Pvu*II from pλDα and cloned into the *Pst*I and *Sma*I sites in pBSC to make pλD5.

For construction of plasmid vectors for the C-terminal fusion of gpD, *Nhe*I and *Eag*I sites were introduced between the *D* and *E* genes by PCR using two pairs of oligonucleotide primers, 5'-CCGGGGATCCTCAACTGT-GAGGA and 5'-AACGGCCGAATGCTAGCGATAAC-GATGCTGATTGCCGTTCCGGC, and 5'-AACGGCC-GCTTTACCCTTCATCACTAAAG and 5'-TGCCCTTA-AGCACGGCAGAAACT, and λ1685 as a template. The two PCR fragments were digested with *Bam*HI and *Eag*I, and *Eag*I and *Afl*II, respectively, and were cloned in tandem between the *Bam*HI and *Afl*II sites in pλDo to create pλDne. The *D* gene ochre termination codon, TAA, was replaced with an amber termination codon, TAG, in pλDne. DNA encoding a collagenase recognition sequence, peptide linker, MCS and *lacZ'* was amplified by PCR from λfoo DNA, using primers, 5'-AAGC-TAGCAGCTGGCCTGTTGGGCCCACT and 5'-AACG-GCCGCTATCTATCTAGAATTCGAGCTCGGTACCCG-GG. This PCR fragment was digested with *Nhe*I and *Eag*I, and cloned between the *Nhe*I and *Eag*I sites in pλDne, resulting in plasmid pλDβ. A *D* gene fragment was also isolated from pλDne with *Bam*HI and *Eag*I and cloned into *Bam*HI and *Eag*I in pBSC. By eliminating MCS

between *Kpn*I and *Bam*HI in pBSC, pλD was created. The PCR fragment encoding a collagenase recognition sequence, peptide linker, MCS and *lacZ'* was inserted into *Nhe*I and *Eag*I in pλD to make pλD3. All oligonucleotide primers used in this work were synthesized using an Applied Biosystem DNA synthesizer model 394 (Foster City, CA). Sequences of all PCR products were confirmed by DNA sequencing (Sanger *et al.*, 1977), since PCR induces mutations at a high rate (Maruyama, 1990; Williams & Winter, 1993).

A 4755 bp *Apa*I-*Apa*LI fragment of pλDα and a 4950 bp *Apa*I-*Apa*LI fragment of pλDβ were cut out with *Apa*I and *Apa*LI, and ligated with left and right arms purified from λfoo DNA digested with *Apa*I and *Apa*LI to make phage vectors, λfooDn and λfooDc, respectively. *In vitro* packaging of the ligated DNAs was carried out as described (Maruyama *et al.*, 1994).

Cloning of genes into vectors

The β-lactamase gene was isolated from pUC18 by PCR amplification, using primers 5'-GAGGTACCCTGGTGA-AAGTAA and 5'-GTGGTACCCTCGAGCCAATGCT-TAATCAGTGA. The amplified DNA was digested with *Kpn*I and cloned into the *Kpn*I site of pλD1, creating pλLD.

Plasmid pRIT5 was used as a template for PCR to amplify a DNA fragment encoding the IgG-binding domains of protein A, using primers 5'-TTCTGCAG-CGCGCAACACGATGAAGCTCAA and 5'-TTGGTAC-CGCTCACCGAAGGATCGTC. The PCR fragment encoding the domains E, D, A and B was digested with *Pst*I and *Kpn*I, and inserted into the *Pst*I and *Kpn*I sites of pλDb to fuse the domains to the N terminus of gpD. To produce the protein A-gpD fusion protein in bacteria, the fragment encoding the protein A-gpD fusion was cut out with *Pst*I and *Pvu*II, and cloned into the *Pst*I and *Sma*I sites of pBSC. The resulting construct was designated pλAD, in which expression of the fusion protein was placed under the control of the *lac* promoter. A DNA fragment encoding the B domain of protein A was cut out with *Hin*dIII and *Eco*RI from pλAD and cloned into the *Hin*dIII and *Eco*RI sites of pλD3, resulting in plasmid pλDA. pAD, a deletion derivative of pλAD, was made by eliminating a 465 bp *Hin*dIII fragment encoding the IgG-binding domains E, D and A of protein A.

For the construction of λPAn, the protein A gene was isolated from pλAD by digestion with *Hin*dIII and *Eco*RI, and cloned into the *Hin*dIII and *Eco*RI sites of λfooDn. Similarly, λPAc was constructed from a *Hin*dIII-*Eco*RI fragment of pλAD and λfooDc digested with *Hin*dIII and *Eco*RI. The β-galactosidase gene was excised from pMC1871 by *Bam*HI and cloned into λfooDc to make λDβ-gal.

Fusion protein expression

gpD and its fusion products were produced intracellularly at a higher level from pBSC-based vectors (pλD, pλAD and pλDA) or at a lower level from pACYC184-based vectors (pλD1 and pλLD). *E. coli* strains harbouring plasmid pλD, pλAD or pλDA were grown in CY medium (Maruyama *et al.*, 1994) containing 10 mM $MgSO_4$ and 100 μg/ml ampicillin, and *E. coli* strains harbouring plasmid pλD1 or pλLD were grown at 37°C overnight in the same medium containing 10 μg/ml chloramphenicol. λF7 was added to a 1.0 ml overnight culture of bacteria at 0.1 multiplicity of infection. After adsorption at 37°C for ten minutes, the culture was diluted 100-fold with CY containing 10 mM $MgSO_4$ and appropriate antibiotics, and incubated with vigorous agitation at 37°C until complete cell lysis. When TG1 was used as a host, culture was diluted with CY containing 10 mM $MgSO_4$, 100 μg/ml ampicillin, and 1.0 mM isopropyl-β,D-thiogalactopyranoside.

Assay of fusion proteins

For the EDTA-sensitivity test, phages were incubated either with TM buffer (10 mM Tris-HCl (pH 7.4), 10 mM $MgSO_4$) or TE buffer (10 mM Tris-HCl (pH 7.4), 10 mM EDTA) for 15 minutes at 37°C and titrated on *E. coli* strain WXO, as described by Sternberg & Weisberg (1977).

The activity of β-lactamase displayed on phage heads was assayed with Nitrocefin (Becton Dickinson, Cockeysville, MD) as a colour indicator (O'Callaghan *et al.*, 1972). For phage purification, CsCl step-gradients were prepared: first layer, 5.95 g CsCl in 3.5 ml of TM buffer; second layer, 3.75 g CsCl in 2.5 ml of TM buffer; third layer, 3.25 g CsCl in 2.5 ml of TM buffer. λF7 phage lysate grown on *E. coli* EQ166 containing pλLD was fractionated through the CsCl step-gradient by centrifugation at 15,000 *g* for 2.4 hours at 4°C. Ten-drop samples were collected from the bottom of the tube. The phage titer of each fraction was measured after incubating in TE buffer for 15 minutes at 37°C, using WXO as a host. β-Lactamase activity was measured as absorbance at 486 nm after the addition of Nitrocefin to the fractions.

The IgG-binding activity of protein A fused to phage heads was tested by its ability to bind to rabbit anti-BSA antibody (Sigma, St Louis, MO). Microtiter wells were coated with 1 mg/ml BSA in PBS (130 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, (pH 7.2)) at 4°C for 12 to 16 hours. The wells were rinsed three times with distilled water, filled with blocking buffer (PBS, 0.05% (v/v) Tween-20, 5% (w/v) skimmed milk, 0.5% (w/v) gelatin) at room temperature for 30 minutes, and then rinsed three times with distilled water. Phage was mixed with antibody in binding buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween-20, 10 mM $MgSO_4$) at 4°C for 12 to 16 hours and applied to the plate. After two hours of incubation at room temperature, a 1 μl aliquot was removed, diluted with λ-dil (Maruyama *et al.*, 1994), and titrated on WXO for infectious phages. Phage adsorbed to the plate was also recovered by collagenase (Sigma) treatment as described by Maruyama *et al.* (1994), and a 1 μl aliquot was also taken from the plate and titrated on WXO. In the experiment of affinity selection of λPAc and λPAn, phage in λ-dil was applied to microtiter wells coated with rabbit anti-BSA antibody (0.22 μg/μl in PBS) at 4°C overnight. Conditions for washing of the plate and elution of phage were as described above.

Assay of β-galactosidase activity was conducted as described by Maruyama *et al.*, (1994).

Western blotting

λPAn grown in 10 ml each of EQ166, MC8 or Q358 were precipitated by the addition of PEG8000 (Fisher) to 10% (w/v) and NaCl to 1.0 M. Pellets were resuspended in 1.0 ml of λ-dil and reprecipitated with PEG as above. After dialysis of the phage suspension against TM, an aliquot of approximately 10^8 phage particles was boiled in sample buffer (62.5 mM Tris-Cl (pH 6.8), 10% (v/v) glycerol, 2.0% (w/v) SDS, 1.0% (v/v) β-mercaptoethanol,

0.005% (w/v) bromophenol blue) and applied to an SDS/12% polyacrylamide gel. Proteins separated by the electrophoresis were electro-transferred to a nitrocellulose filter as described by Maruyama *et al.* (1994). Protein A fused to gpD was detected by staining the blot with rabbit anti-BSA primary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody, and BCIP/NBT (BIO-RAD) as chromogenic substrates.

Acknowledgements

The authors are grateful to Nalin Kumar and Roberto Jappelli for their reading of the manuscript. This work was initiated in the Department of Medicine, University of Cambridge, with support of the Louis Jeantet Research Foundation. The work at The Scripps Research Institute was supported by the Markey Charitable Trust, a US NSF grant, and TAITEC Corp.

References

Casadaban, M. J., Martinez-Arias, A., Shapira, S. K. & Chou, J. (1983). β-Galactosidase gene fusions for analysing gene expression in Escherichia coli and yeast. *Methods Enzymol.* **100**, 293–308.

Chang, A. C. Y. & Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* **134**, 1141–1156.

Dokland, T. & Murialdo, H. (1993). Structural transitions during maturation of bacteriophage lambda capsids. *J. Mol. Biol.* **233**, 682–694.

Dunn, I. S. (1995). Assembly of functional bacteriophage lambda virions incorporating C-terminal peptide or protein fusions with the major tail protein. *J. Mol. Biol.* **248**, 497–506.

Germino, J. & Bastia, D. (1984). Rapid purification of a cloned gene product by genetic fusion and site-specific proteolysis. *Proc. Natl Acad. Sci. USA*, **81**, 4692–4696.

Hart, S. L., Knight, A. M., Harbttle, R. P., Mistry, A., Hunger, H. D., Cutler, D. F., Williamson, R. & Coutelle, C. (1994). Cell binding and internalization by filamentous phage displaying a cyclic Arg-Gly-Asp-containing peptide. *J. Biol. Chem.* **269**, 12468–12474.

Imber, R., Tsugita, A., Wurtz, M. & Hohn, T. (1980). Outer surface protein of bacteriophage lambda. *J. Mol. Biol.* **139**, 277–295.

Jespers, L. S., Messen, J. H., Keyser, A. D., Eeckhout, D., Brande, I. V. D., Gansemans, Y. G., Lauwereys, M. J., Vlasuk, G. P. & Stanssens, P. E. (1995). Surface expression and ligand-based selection of cDNAs fused to filamentous phage gene VI. *Bio/Technology*, **13**, 378–382.

Karn, J., Matthes, H. W. D., Gait, M. J. & Brenner, S. (1984). A new selective phage cloning vector, λ2001, with sites for *Xba*I, *Bam*HI, *Hin*dIII, *Eco*RI, *Sst*I and *Xho*I. *Gene*, **32**, 217–224.

Kasahara, N., Dozy, A. M. & Kan, Y. W. (1994). Tissue-specific targeting of retroviral vectors through ligand-receptor interactions. *Science*, **266**, 1373–1376.

Lu, Z., Murray, K. S., Cleave, V. V., LaVallie, E. R., Stahl, M. L. & McCoy, J. M. (1995). Expression of thioredoxin random peptide libraries on the *Escherichia coli* cell surface as functional fusions to flagellin: A system designed for exploring protein-protein interactions. *Bio/Technology*, **13**, 366–372.

Maina, C. V., Riggs, P. D., Grandea, A. G., III, Slatko, B. E., Moran, L. S., Tagliamonte, J. A., McReynolds, L. A. & Guan, C. D. (1988) An *Escherichia coli* vector to express and purify foreign proteins by fusion to and separation from maltose-binding protein. *Gene*, **74**, 365–373.

Maruyama, I. N. (1990). Estimation of error rate in the polymerase chain reaction. *Technique*, **2**, 302–303.

Maruyama, I. N. & Brenner, S. (1992). A selective λ phage cloning vector with automatic excision of the insert in a plasmid. *Gene*, **120**, 135–141.

Maruyama, I. N., Maruyama, H. I. & Brenner, S. (1994). λfoo: a λ phage vector for the expression of foreign proteins. *Proc. Natl Acad. Sci. USA*, **91**, 8273–8277.

Nilsson, B., Abrahmsén, L. & Uhlén, M. (1985). Immobilization and purification of enzymes with staphylococcal protein A gene fusion vectors. *EMBO J.* **4**, 1075–1080.

O'Callaghan, C. H., Morris, A., Kirby, S. M. & Shingler, A. H. (1972). Novel method for detection of beta-lactamase by using a chromogenic cephalosporin substrate. *Antimicrob. Agent & Chemother.* **4**, 283–288.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487–491.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, **228**, 1315–1317.

Sternberg, N. & Hoess, R. H. (1995). Display of peptides and proteins on the surface of bacteriophage λ. *Proc. Natl Acad. Sci. USA*, **92**, 1609–1613.

Sternberg, N. & Weisberg, R. (1977). Packaging of coliphage lambda DNA. II. The role of the gene *D* protein. *J. Mol. Biol.* **117**, 733–759.

Uhlén, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L. & Lindberg, M. (1984). Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. *J. Biol. Chem.* **259**, 1695–1702.

Wells, J. A. & Lowman, H. B. (1992). Rapid evolution of peptide and protein binding properties in vitro. *Curr. Opin. Biotechnol.* **3**, 355–362.

Williams, S. C. & Winter, G. (1993). Cloning and sequencing of human immunoglobulin V_λ gene segments. *Eur. J. Immunol.* **23**, 1456–1461.

Willis, A. E., Perham, R. N. & Wraith, D. (1993). Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage. *Gene*, **128**, 79–83.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, **33**, 103–119.

Edited by J. Karn

(Received 14 June 1996; accepted 11 July 1996)